Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 286 (2005) 549 – 558

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Genetic dissection of trophic interactions in the larval optic neuropil of *Drosophila melanogaster*

Verónica G. Rodriguez Moncalvo, Ana Regina Campos *

Department of Biology, McMaster University, 1280 Main St. West, Hamilton, ON, Canada L8S 4K1

Received for publication 13 June 2005, revised 17 August 2005, accepted 18 August 2005

Abstract

The larval visual system of *Drosophila melanogaster* consists of two bilateral clusters of 12 photoreceptors, which express Rhodopsin 5 and 6 (Rh5 and Rh6) in a non-overlapping manner. These neurons send their axons in a fascicle, the larval optic nerve (LON), which terminates in the larval optic neuropil. The LON is required for the development of a serotonergic arborization originating in the central brain and for the development of the dendritic tree of the circadian pacemakers, the small ventral lateral neurons (LNv) [Malpel, S., Klarsfeld, A., Rouyer, F., 2002. Larval optic nerve and adult extra-retinal photoreceptors sequentially associate with clock neurons during *Drosophila* brain development. Development 129, 1443–1453; Mukhopadhyay, M., Campos, A.R., 1995. The larval optic nerve is required for the development of an identified serotonergic arborization in *Drosophila melanogaster*. Dev. Biol., 169, 629–643]. Here, we show that both Rh5- and Rh6-expressing fibers overlap equally with the 5-HT arborization and that it, in turn, also contacts the dendritic tree of the LNv. The experiments described here aimed at determining whether Rh5- or Rh6-expressing fibers, as well as the LNv, influence the development of this serotonergic arborization. We conclude that Rh6-expressing fibers play a unique role in providing a signal required for the outgrowth and branching of the serotonergic arborization. Moreover, the innervation of the larval optic neuropil by the 5-HT arborization depends on intact Rac function. A possible role for these serotonergic processes in modulating the larval circadian rhythmicity and photoreceptor function is discussed.

Keywords: Rhodopsin; Photobehavior; *Drosophila*; Serotonin; Larva; Mutant; Photoreceptors; Pacemaker

Introduction

Assembly of neural circuits requires the orchestration of multiple intrinsic and extrinsic signals (for reviews, see Grueber and Jan, 2004; Jan and Jan, 2003; McAllister, 2000). For instance, the role of neurotrophins in dendrite development and maintenance is well established (Huang and Reichardt, 2001; McAllister, 2001; McAllister et al., 1995). Recently, receptor–ligand systems initially identified on the basis of their function in axon guidance have been added to the growing list of extrinsic cues that modulate dendrite development (Furrer et al., 2003; Polleux et al., 2000; Whitford et al., 2002). Furthermore, the role of afferent neurotransmission in dendritic development is also well established and conserved in many organisms (Li et al., 2002; Rajan and Cline, 1998; Rajan et al., 1999; Sin et al., 2002; Wong and Ghosh, 2002).

Although the underlying mechanisms by which extrinsic factors exert their effects on dendritic and axonal growth remain largely unknown, accumulating evidence in different model systems has revealed links between some of these factors and the activation of Rho GTPases (Fan et al., 2003; Hu et al., 2001; Li et al., 2002; Sin et al., 2002; Yamashita et al., 1999). Moreover, these studies support the notion that members of the Rho family of GTPases function as key integrators of extrinsic and intrinsic cues that regulate the underlying dendritic and axonal cytoskeleton.

This report examines the interaction among three groups of neural processes in the *Drosophila melanogaster* larval optic neuropil. The central nervous system of *Drosophila* like in many other insects follows a typical organization in which the somata of neurons and glia form an outer layer surrounding the inner neuropil where axons and dendrites are segregated (Campos et al., 1995; Nassif et al., 2003). By the end of embryogenesis, the larval optic nerve (LON) terminates within the optic lobe anlagen in the larval optic neuropil area. The larval visual system

* Corresponding author. Fax: +1 905 522 6066.
E-mail address: camposa@mcmaster.ca (A.R. Campos).

0012-1606/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2005.08.030

of *Drosophila* consists of a pair of bilateral visual organs also known as Bolwig's organs, situated just anterior to the cephalopharyngeal skeleton (Green et al., 1993; Steller et al., 1987). Each of these visual organs is made up of 12 photoreceptor cells that differentiate during stage 13 of embryogenesis (Campos et al., 1995; Green et al., 1993) and are divided in 2 subsets: ~4 cells expressing the blue-absorbing opsin Rh5 (λ_{max} = 437 nm) and ~8 cells expressing the green-absorbing opsin Rh6 (λ_{max} = 508 nm) (Malpel et al., 2002).

It has been previously shown that the LON overlaps with a serotonergic arborization that originates from cell bodies located in the central brain (Mukhopadhyay and Campos, 1995). More recently, it has been reported that the LON terminus overlaps with the dendritic arborization of a subset of circadian pacemaker neurons, the small ventral lateral neurons (LNv) (Malpel et al., 2002). Interestingly and similar to what was previously shown for the serotonergic arborization, the development of the LNv dendritic arbor is dependent on contact with the LON (Malpel et al., 2002; Mukhopadhyay and Campos, 1995).

Here, we show that both Rh5- and Rh6-expressing fibers overlap with the 5-HT arborization, which in turn, also contacts the dendritic tree of the LNv. The results of cell ablation experiments indicate that the presence of Rh6-expressing fibers is necessary for the development of the serotonergic arborization. Moreover, suppression of synaptic activity by targeted expression of tetanus toxin light chain (TNT) in Rh6-expressing fibers prevents the full development of this 5-HT arborization, suggesting that this is at least in part an activity-dependent process. Finally, our results implicate Rac signaling in the development of the serotonergic arborization.

Materials and methods

Fly stocks

All *D. melanogaster* stocks were raised at 25°C in standard medium containing inactivated yeast, sucrose, agar, 10% and tegosept in ethanol to prevent mold growth. Cell ablation was achieved by targeted expression of the cell death genes *head involution defective* (*hid*) or *reaper* (*rpr*) using the *Drosophila* stocks *yw; P[UAS-hid]/P[UAS-hid]*) (Grether et al., 1995) and *+/+;P[UAS-rpr]/P[UAS-rpr]* (White et al., 1996) respectively. Neuronal silencing was achieved by targeted expression of tetanus toxin light chain (*TNT*) or non-inactivating Shaker K^+ channel protein (electrically knock out or EKO) (White et al., 2001). Three forms of *TNT* were used: the active forms *w; P[UAS-TNT-E]* and *w; P[UAS-TNT-G]* and the inactive control *w; P[UAS-TNT-VIF]* (Sweeney et al., 1995). The strain *GMR-hid* was used to ablate all photoreceptors (Grether et al., 1995). *GMR-Gal4* (Bloomington Stock Center, Indiana University, IN, #1104), *Rh6-Gal4* and *Rh5-Gal4* transgenic strains allowed expression of the target genes in all larval photoreceptors, Rh6- or Rh5-expressing larval photoreceptors cells respectively. In the case of the *Rh6-Gal4* and *Rh5-Gal4* strains, the co-expression of *P[UAS-mCD8:GFP]* construct (Bloomington Stock Center, Indiana University, IN, #5137) allowed the visualization of Rh5 and Rh6 termini. In addition, a *Ddc-Gal4* line (HL836, third chromosome) kindly provided by Jay Hirsch (University of Virginia, VA) and the *P [UAS-mCD8: GFP]* construct (Bloomington Stock Center, Indiana University, IN, #5130) were used to recombine both transgenes in the same chromosome and to target the expression of the green fluorescent protein (GFP) in the serotonergic cells. Similarly, *yw; P [PDF-Gal4]* and the *P [UAS-mCD8: GFP]* construct (Bloomington Stock Center, Indiana University, IN, #5137) were recombined to express GFP specifically in the LNv. Standard wild type stock *Oregon-R* (*OR*) was used. For *Rac* mutant analysis, the *Rac2*-specific null mutant homozygous viable $Rac2^{\Delta}$ *ry* stock, the *yw;* $Rac1^{J11}$ *FRT2A/TM6B* stock (which contains a null allele copy of $Rac1^{J11}$) and the *yw;* $Rac1^{J11}$ $Rac2^{\Delta}$ *FRT2A/TM6B* stock were utilized (Bloomington Stock Center, Indiana University, IN, #6675, 6674 and 6677 respectively).

Histology, immunohistochemistry and imaging

Late wandering third instar larval brains were dissected, fixed and incubated with the appropriate primary antibody according to a previously published protocol (Mukhopadhyay and Campos, 1995). In order to visualize photoreceptor axons, the mouse monoclonal antibody anti-CHAOPTIN (24B10, 1:100), which recognizes CHAOPTIN, a glycoprotein expressed specifically on the photoreceptor cell plasma membrane, was used (Van Vactor et al., 1988; Zipursky et al., 1984). 5-HT neurons were labeled using rabbit anti-serotonin (1:200) (Protos Biotech Corp., NY). Accordingly, the secondary antibodies used were Alexa 488-conjugated goat anti-mouse IgG (1:200) (Molecular Probes Inc., Eugene, OR) and Texas Red-conjugated goat anti-rabbit IgG (1:200) (Jackson InmunoResearch Laboratories, Inc., West Grove, PA). The specimens were viewed in a Nikon Eclipse ∈800 microscope. Confocal images were obtained with a Bio-Rad Radiance MRC 600 Krypton/Argon laser confocal microscope using the LaserSharp software. Each image consisted of *z*-stack of 2 to 25 sections approximately at 1 μm intervals, and they were adjusted for brightness and contrast using Adobe Photoshop 5.0 for Macintosh. In the case of cell ablation and neuronal silencing, all specimens were first analyzed blind.

Behavioral assays

Third instar foraging larvae at 84–90 h after egg laying (AEL) were harvested following the protocol described previously (Busto et al., 1999). Photobehavioral assays were carried out using a semi-automated assay system previously used in our laboratory (Busto et al., 1999; Hassan et al., 2005). Briefly, individual larvae were placed on a test arena of non-nutritive agar and were exposed to alternative 10-s pulses of light and dark for a total of at least 60 s. The tracking program controlled the periodicity of the light stimulus while allowing a stylus/tablet-based tracking of larval locomotion. The light stimulus was controlled by a serial device MacIO microcontroller (MacBrick, Netherlands) and by a relay to obtain a 10-s periodicity of the light pulse. At the end of each assay, the macro automatically calculated a response index, RI = [(total distance traveled in the dark period − total distance traveled in the light period) / total distance traveled in both the periods]. Since the response to light in this assay depends on the ability of the larva to move efficiently, larval locomotion in constant darkness was measured as a control.

Statistical analysis

Minitab 10.5 Xtra for Macintosh was used in the statistical analysis of samples. Statistical tests employed in the analysis of data included one-way analysis of variances (ANOVAs), Tukey–Kramer post-hoc multiple comparison tests and normality test on the residuals of ANOVA using Rootogram test.

Results

Both the Rh5- and the Rh6-expressing fibers overlap with the larval optic neuropil 5-HT arborization

The LON is formed by two groups of axons distinguished by the non-overlapping expression of Rh5 and Rh6 (Malpel et al., 2002). In late third instar larvae, the LON is found intimately associated with a 5-HT arborization in the larval optic center (Figs. 1A–C and Mukhopadhyay and Campos, 1995). However, whether both sets of photoreceptor cells are involved in this contact was not known. In order to address this question, brains dissected from wandering third instar larvae in which the Rh5-

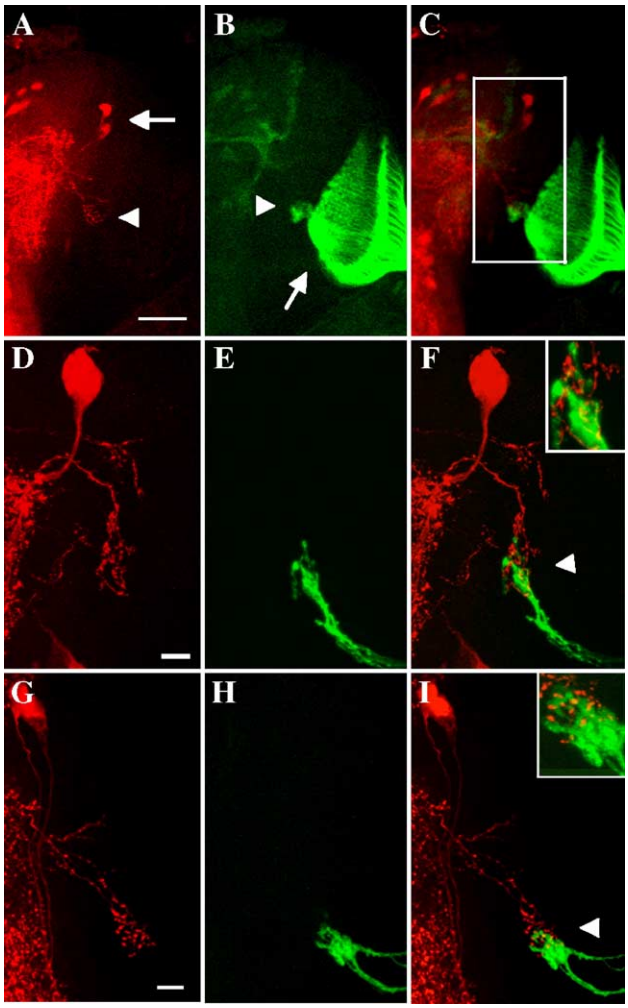

Fig. 1. The termini of Rh5 and Rh6 expressing photoreceptors overlap with a 5-HT arborization in the larval optic neuropil. (A–C) Low magnification confocal micrographs of a wild type wandering third instar larval hemisphere immunolabeled with anti-5-HT detected by Texas Red-conjugated secondary (red in panel A and in all subsequent panels and figures) and 24B10 monoclonal antibody detected by Alexa 488-conjugated secondary (B, green), showing the relationship between the LP1 serotonergic cell bodies (arrow in panel A), the serotonergic arborization in the larval optic center (arrowhead in panel A), the LON (arrowhead in panel B) and the developing adult retinal projection (arrow in panel B). (C) Merge of panels A and B. The box represents the area studied in all panels and subsequent figures. (D–I) High magnification confocal micrographs of GFP expression in Rh5 or Rh6 specific photoreceptors by targeted expression of the *UAS-CD8-GFP* construct using either the *Rh5-Gal4* or *Rh6-Gal4* drivers (D–F, *UAS-CD8-GFP; Rh5-Gal4*; G–I, *UAS-CD8-GFP; Rh6-Gal4*). (D) The LP1 cell bodies lie near the 5-HT arborization in the larval optic neuropil. (E) The termini of the Rh5-expressing photoreceptor axons in the same region. (F) Merge of panels D and E showing the overlapping between the 5-HT arborization and the Rh5 photoreceptors termini (arrowhead). The inset shows a higher magnification of this region. (G–I) Same as in panels D–F but showing the overlap of the Rh6 photoreceptor termini with the 5-HT arborization in the larval optic neuropil (arrowhead in panel I). Scale bar in panel A represents 40 μm and is valid for panels B and C. Scale bar in panels D and G represents 10 μm and is valid for panels E, F, H and I.

specific photoreceptors neurons express CD8-GFP under the regulation of the *Rh5* promoter (*yw; UAS-CD8-GFP; Rh5-GAL4*, $N = 18$) were labeled with 5-HT antibody (Figs. 1D–F). Similarly, aged specimens dissected from strains in which CD8-GFP was specifically expressed in Rh6 neurons were labeled with 5-HT (*w; UAS-CD8-GFP; Rh6-GAL4*, $N = 18$) (Figs. 1G–I). At the level of resolution afforded by the confocal microscope, while variable from specimen to specimen, the termini of either subset of photoreceptor axons did not display any distinguishing characteristics. Likewise, the degree of overlap between both subset of photoreceptor axons and the 5-HT arborization in the larval optic neuropil was apparently the same (Figs. 1F and I).

The 5-HT arborization is intimately associated with the dendritic tree of the LNv in the larval optic neuropil

It has been previously reported that the larval LNv contact both the Rh5- and the Rh6-expressing fibers and that this interaction with the LON provides trophic support for the development of the LNv dendritic tree (Malpel et al., 2002). We asked whether the LNv would also contact the serotonergic arborization in the larval optic center. To that end, larval brains in which the LNv were labeled by virtue of the targeted expression of CD8-GFP (*yw; PDF-GAL4, UAS-CD8-GFP*, $N = 11$) were treated with 5-HT antibody (Fig. 2). As shown in Fig. 2C, the 5-HT arborization was found in intimate association with the dendritic tree of the larval LNv (arrowhead).

The development of the serotonergic arborization does not depend upon the presence of the Rh5-expressing fibers or the larval LNv

In order to determine whether the Rh5-expressing fibers influence the development of the 5-HT arborization, we investigated the impact of absence of Rh5 fibers on the integrity of the serotonergic arborization as seen by anti 5-HT

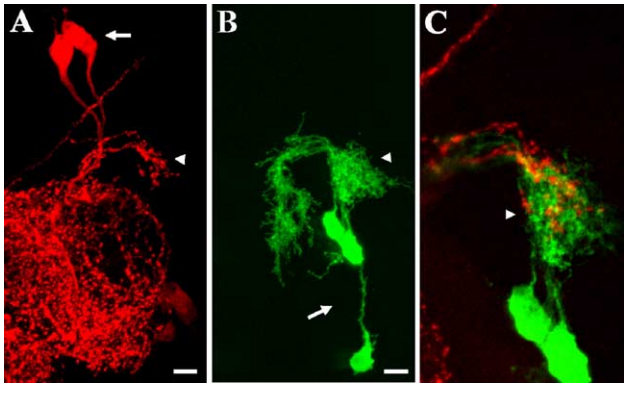

Fig. 2. The dendritic arborization of the LNv overlaps with the serotonergic innervation of the larval optic neuropil. Confocal micrographs of brains dissected from wandering third instar larvae (*PDF-Gal4, UAS-CD8-GFP*) in which GFP (green) expression is targeted to the main circadian pacemakers, the LNv, and labeled with anti 5-HT antibody (red) and detected as mentioned before. (A) LP1 serotonergic cells (arrow) and the larval optic neuropil 5-HT arborization (arrowhead). (B) LNv (arrow) and their dendritic tree (arrowhead) in the larval optic center. (C) Higher magnification merge of panels A and B, showing the overlap between the LNv dendrites and the 5-HT arborization. Scale bars: 10 μm.

immunolabeling. For this purpose, the Rh5-expressing photoreceptors were ablated by targeted expression of the cell death gene *hid* under the regulation of the *Rh5-GAL4* driver. In this and all subsequent experiments, cell ablation was assessed by the concomitant expression of the CD8-GFP reporter or by photoreceptor-specific protein CHAOPTIN staining (Zipursky et al., 1984). As shown in Fig. 3B, no obvious defect in the 5-HT arborization (arrowhead) is observed when the Rh5-expressing photoreceptors are absent (*yw; UAS-CD8-GFP/UAS-hid; Rh5-GAL4/+*, $N = 31$), suggesting that this subset of photoreceptor cells is not required for the proper innervation of the larval optic neuropil by the serotonergic arborization. A similar result was observed when the LNv were ablated due to targeted expression of *rpr* death gene by *PDF-GAL4*. In these specimens, the serotonergic arborization is indistinguishable from that of wild type samples, suggesting that it develops normally in the absence of the LNv (Fig. 3D, arrowhead) (*UAS-CD8-GFP, PDF-GAL4/UAS-rpr*, $N = 14$).

Rh6-expressing fibers are required for the development of the serotonergic arborization

In order to examine whether the Rh6-expressing fibers are required for normal development of the serotonergic arborization, the Rh6 cells and their axons were ablated by targeted expression of *hid* driven by the *Rh6-GAL4* element. No 5-HT labeling was detected in the larval optic neuropil area when the Rh6 fibers were absent (Fig. 4 compare A to C) (*UAS-CD8-GFP/UAS-hid; Rh6-GAL4/+*, $N = 26$). These results are indistinguishable from those obtained by the complete absence of the LON in *glass* mutants as previously reported by Mukhopadhyay and Campos (1995) or due to the expression of *hid* under the *glass multimer reporter (GMR)* promoter (Fig. 4B, $N = 16$). Thus, these observations suggest that, among the larval photoreceptors, the presence of the Rh6-expressing fibers is specifically required for the development of the serotonergic arborization.

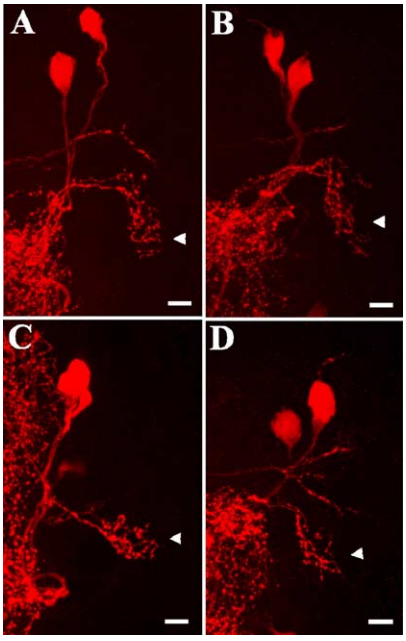

Fig. 3. Ablation of the Rh5-specific photoreceptors or LNv does not affect the development of the 5-HT arborization. The Rh5 photoreceptors or the LNv were ablated by targeted expression of the dead genes *hid* or *rpr* respectively, and the dissected brains were labeled with 5-HT antibody as described before. In all specimens, ablation was nearly complete as determined by the absence of expression of GFP in the targeted cells. (A) Parental strain (*UAS-CD8-GFP/+*; *Rh5-Gal4/+*) showing the stereotypical location of the 5-HT arborization (arrowhead). (B) *UAS-CD8-GFP/UAS-hid*; *Rh5-Gal4/+*. Ablation of Rh5 photoreceptors has no apparent effect in the development of the 5-HT arborization (arrowhead). (C) Parental strain (*PDF-Gal4, UAS-CD8-GFP/+*) showing the stereotypical location of the 5-HT arborization (arrowhead). (D) *PDF-Gal4, UAS-CD8-GFP/UAS-rpr*. Similar to what is observed for the Rh5 photoreceptors, the LNv are not required for the development of the 5-HT arborization (arrowhead). Scale bars: 10 μm.

Simultaneous ablation of Rh5 photoreceptors and LNv does not disrupt the development of the 5-HT arborization

The results presented above do not address whether Rh6 projection is sufficient for the development of the 5-HT arborization in the larval optic neuropil. In order to assess this, one would need to eliminate all other neurons known to project to the larval optic neuropil except the Rh6 photoreceptor cells. While the identity of all neurons that project to the larval optic center is not known, two other types of neurons, namely the Rh5 photoreceptors and the LNv, have been shown not to be required individually for the presence of 5-HT labeling in the larval optic center. Given that there are only 3–4 Rh5 axons and a similar number of LNv axons, it is possible that reduction of a putative trophic support provided by either Rh5 or LNv alone is not sufficient to impact the development of the 5-HT arborization. In order to address this question and to determine whether the requirement of Rh6 projection for the development of the 5-HT arborization in the larval optic neuropil is a feature unique of these neurons, we ablated Rh5 photoreceptors and LNv simultaneously by the targeted expression of *rpr* as described above. No obvious defect in the 5-HT arborization is observed when both the LNv and the Rh5-expressing fibers are absent (arrowhead in Fig. 5B) (*PDF-GAL4, UAS-CD8-GFP/UAS-rpr; Rh5-Gal4/+*, $N = 16$). Thus, these results further confirm that the larval LNv and Rh5-expressing fibers are not required for the normal development of the 5-HT arborization.

Absence of 5-HT arborization in LON ablated larvae is due to reduced branching and not lack of 5-HT expression

Disruption in the development of the serotonergic arborization as a consequence of afferent ablation is inferred by the absence of 5-HT immunolabeling in the larval optic neuropil. As such, these results do not distinguish between reduction in 5-HT synthesis and/or transport or impaired branching of these neurons. In order to distinguish between these alternatives, we sought to visualize these neurons and their projections by targeting the expression of GFP using a Gal4 driver regulated by the *Dopa decarboxylase* (*Ddc*) gene regulatory region (Li et al., 2000). In these larvae, serotonergic as well as dopaminergic neurons and their projections can be visualized

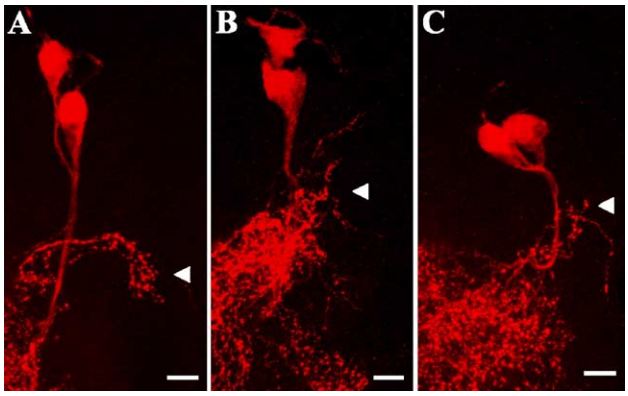

Fig. 4. The Rh6 photoreceptors are required for the proper development of the 5-HT arborization. (A) Wild type parental control (*UAS-CD8-GFP/+; Rh6-Gal4/+*) showing the stereotypical 5-HT innervation of the larval optic neuropil (arrowhead). (B) *GMR-hid*. Ablation of the larval eye by the targeted expression of the cell death gene *hid* in all photoreceptors causes a significant reduction in the 5-HT arborization (arrowhead). (C) *UAS-CD8-GFP/UAS-hid; Rh6-Gal4/+*. Ablation of all Rh6 photoreceptors by targeted expression of *hid* in these cells causes a similar reduction in the serotonergic arborization (arrowhead). Scale bars: 10 μm.

by virtue of GFP expression driven by the *Ddc* gene promoter. The GFP-expressing projection that corresponds to the serotonergic arborization in the larval optic neuropil can be identified due to its stereotypic position relative to other landmarks and its intimate association with the terminus of the larval optic nerve (Figs. 6A–C). Ablation of larval photoreceptors by expression of the cell death gene *hid* in these larvae appears to impair the branching of the 5-HT arborization as seen by the absence of GFP as well as 5-HT labeling (Figs. 6D–F). These results demonstrate that the LON provides a putative trophic signal required for the branching of this arborization rather than for the expression and localization of 5-HT.

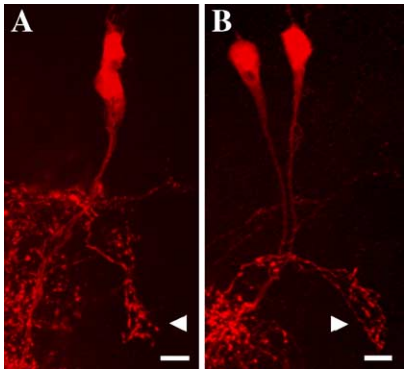

Fig. 5. Normal development of the larval optic neuropil 5-HT arborization in the absence of both the LNv and the Rh5-expressing fibers. Both A and B panels depict confocal micrographs of third instar larval brains labeled with anti 5-HT antibody and detected as before (red). (A) Brain dissected from parental strain (*PDF-Gal4, UAS-CD8-GFP/+; Rh5-Gal4/+*) showing the stereotypical location of the 5-HT arborization (arrowhead). (B) *PDF-Gal4, UAS-CD8-GFP/UAS-rpr; Rh5-Gal4/+*. The development of the 5-HT arborization is normal (arrowhead) in the absence of both the LNv and the Rh5 photoreceptors. Scale bars: 10 μm.

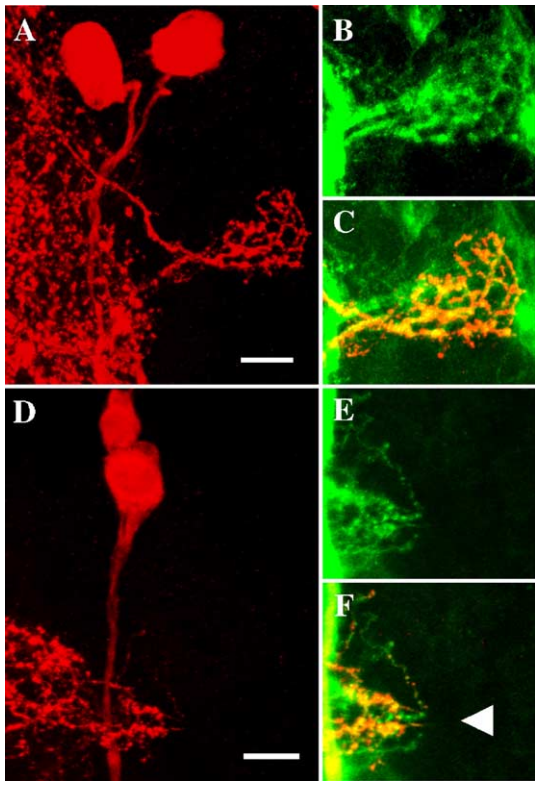

Fig. 6. Absence of 5-HT arborization in LON ablated larvae is due to reduced branching and not lack of 5-HT expression. In order to visualize the development of the serotonergic arborization independently from the expression of 5-HT, a *Ddc-Gal4* driver was used to target GFP expression to these neurons. Panels A–C depict the wild type parental control specimen (*Ddc-Gal4, UAS-CD8-GFP*) showing the expected 5-HT arborization in the larval optic neuropil (A) and Ddc-regulated GFP expression in the same structure (B and C). Ablation of all photoreceptors in these flies by introducing the *GMR-hid* construct (*GMR-hid; Ddc-Gal4, UAS-CD8-GFP*) dramatically reduces this arborization as seen by the lack of *Ddc*-driven GFP expression (D) as well as 5-HT staining (E and F) (arrowhead). Scale bars: 10 μm.

Suppression of synaptic activity in the Rh6-expressing fibers disrupts the branching of the 5-HT arborization

In an attempt to investigate whether synaptic activity of Rh6-expressing fibers may influence the development of the 5-HT arborization, a weak tetanus-toxin light chain allele (TNT-E) or a strong tetanus-toxin light chain allele (TNT-G) was expressed under the control of *Rh6-GAL4* driver. The TNT gene product cleaves synaptobrevin, thereby inhibiting synaptic vesicle docking (Sweeney et al., 1995). In CNS specimens dissected from larvae in which Rh6 photoreceptors expressed TNT-E, a blind analysis revealed that 37.9% of the lobes displayed a notable alteration in the branching of the 5-HT arborization (N = 56, data not shown). A more penetrant phenotype was observed when these cells expressed the stronger TNT-G allele. In these specimens, 79.3% of the lobes displayed a similar reduction of the 5-HT branching as seen when the less active form of TNT was expressed (Fig. 7D, N = 30). Comparable results were obtained when TNT-E or TNT-G were expressed under the control of general photoreceptor driver *GMR-GAL4*. In these specimens, 39.7% of the *GMR-Gal4xUAS-TNT-E* (N = 26; data not shown) and 72.7% of the

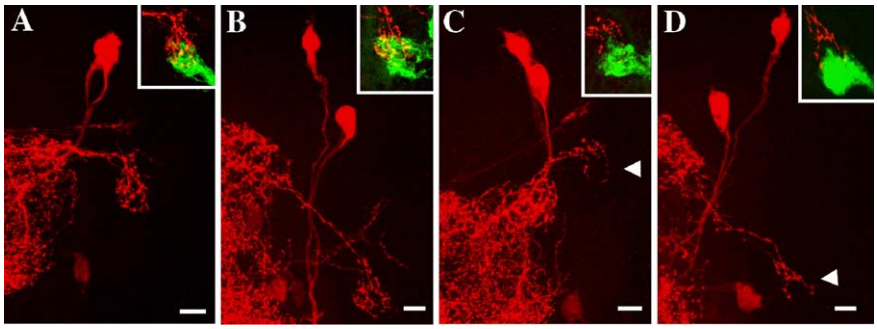

Fig. 7. Suppression of synaptic activity in the Rh6-expressing fibers disrupts the branching of the 5-HT arborization. In order to determine whether the induction of the 5-HT arborization by the larval Rh6 photoreceptor axons is an activity-dependent process, tetanus-toxin light chain (TNT) that suppresses synaptic activity was expressed in all larval photoreceptors or in the Rh6 photoreceptors only. The morphology of the Rh6 photoreceptor termini or the LON terminus was largely normal as evaluated by the concomitant expression of GFP or 24B10 monoclonal antibody staining detected by Alexa 488-conjugated secondary (green) respectively (insets). (A) Wild type parental control *GMR-Gal4/+*. (B) Control in which an inactive form of TNT is expressed in all photoreceptors (*GMR-Gal4/UAS-TNT-VIF*). (C) Expression of TNT-G in all photoreceptors (*GMR-Gal4/UAS-TNT-G*) reduces the extent of 5-HT arborization (arrowhead). (D) A similar phenotype is seen (arrowhead) when expression of TNT-G is restricted to Rh6 photoreceptors only (*UAS-CD8-GFP/UAS-TNT-G; Rh6-Gal4/+*). Scale bars: 10 μm.

GMR-Gal4xUAS-TNT-G lobes (Fig. 7C; $N = 22$) showed such 5-HT branching disruption. In all these cases, the larval photoreceptors developed normally as determined by the pattern of photoreceptor-specific protein CHAOPTIN staining (Zipursky et al., 1984) (Fig. 7, insets).

In order to dissect the role of electrical versus synaptic activity in the development of the serotonergic arborization, we expressed a genetically modified Shaker K^+ channel (the EKO channel) under the control of the *Rh6-GAL4* element. This K^+ channel attenuates electrical activity by being activated at potentials close to E_k and by remaining open (White et al., 2001). Confocal micrographs of third instar larval brains labeled with 5-HT antibody did not reveal any impact on the development of the 5-HT arborization due to the expression of the EKO channels in the Rh6 cells ($N = 27$; data not shown). A similar result was observed when the EKO channel was expressed in all larval photoreceptor cells through the use of the *GMR-Gal4* driver ($N = 14$; data not shown). It is possible that partial suppression of excitability was achieved by expression of these modified channels in these cells. However, behavioral assays carried out with third instar *GMR-GAL4/UAS-EKO* larvae revealed a significant reduction in the response to light compared to controls and as revealed by their mean RIs (*GMR-Gal4/UAS-EKO*, $N = 14$, RI = 0.11; *UAS-EKO/+*, $N = 13$, RI = 0.40; OR, $N = 16$, RI = 0.34; ANOVA: $F_{(2,40)} = 41.51$, $P < 0.001$). Taken together, these results suggest that the innervation of the larval optic neuropil by the 5-HT arborization does not depend on evoked synaptic activity of the Rh6-expressing fibers. Moreover, these results support the notion that spontaneous synaptic activity is sufficient to induce the branching of this serotonergic arborization.

Rac signaling is required for the branching of the 5-HT arborization

While the identity of the LON-derived signal is not known yet, results obtained in other model systems point to Rac GTPases as possible integrators in the activity-dependent development of the serotonergic arborization. Therefore, we investigated the integrity of this projection in larvae with reduced *Rac* function. To this end, we took advantage of a mutant chromosome carrying null mutations in two *Rac* genes (*Rac1* and *Rac2*) present in the *Drosophila* genome (Hakeda-Suzuki et al., 2002; Ng et al., 2002). Heterozygotes carrying at least one wild type copy of either one of the *Rac* genes survive until after the third instar larval stage. Moreover, organisms homozygous for just the *Rac2* null allele are viable. The level of reduction in Rac signaling afforded by these heteroallelic combinations did not cause any major developmental defect in the third instar larval brain as seen by the normal overall 5-HT staining (data not shown). Similarly, the larval photoreceptors developed appropriately as determined by the pattern of photoreceptor-specific protein CHAOPTIN staining (Zipursky et al., 1984) (Fig. 8, insets).

Therefore, we reasoned that the residual *Rac* function provided by one wild-type copy of *Rac1* or *Rac2* was sufficient

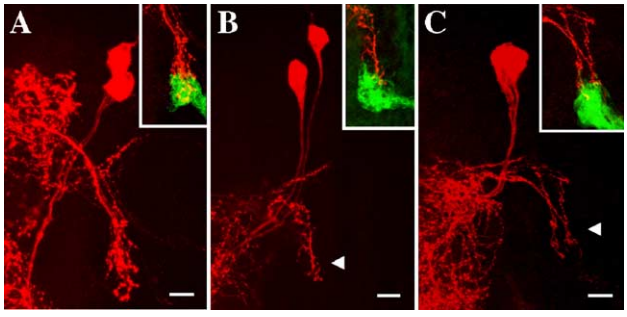

Fig. 8. Induction of 5-HT arborization by the larval optic nerve depends on Rac signaling. Brains from larvae in which Rac function was reduced by mutations in either one of the two Rac genes were dissected and processed for 5-HT labeling as before. The morphology of the larval optic nerve was normal as visualized using the photoreceptor specific 24B10 monoclonal antibody (green). The following panels depict representative confocal micrographs. (A) Wild type control *OR*. (B) *Rac1 Rac2/Rac2* mutant brain. (C) *Rac1 Rac2/Rac1* mutant brain. As seen in panels B and C (arrowheads), both mutants show a reduction in the branching of the serotonergic arborization. The insets in panels B and C show a higher magnification of the 5-HT arborization as its reduced overlap with the LON. Scale bars: 10 μm.

for most of the basic developmental processes required for the assembly of the larval circuitry that takes place earlier during embryogenesis. However, it was possible that inductive processes, such as that which takes place in the larval optic neuropil, may require Rac signaling which is above the level of that provided by the heteroallelic combinations as described before.

The integrity of the 5-HT arborization and the degree of overlap with the LON were analyzed in blind experiments. Fig. 8 depicts representative confocal micrographs of these specimens. Development of the 5-HT arborization was markedly reduced in 83% of CNSs dissected from larvae carrying only one functional copy of either *Rac1* ($N = 42$) or *Rac2* ($N = 22$) (Figs. 8B, C). While the degree of reduction of the 5-HT arborization was similar in all mutant combinations analyzed, the penetrance of this phenotype varied considerably. Marked disruption in the 5-HT arborization was seen in 37% of the *Rac2/Rac2* CNSs (data not shown). Interestingly, the reduction of the 5-HT arborization caused by reduced Rac function is similar to that found in larvae in which synaptic transmission was suppressed by the targeted expression of *TNT* (Fig. 7) and less than that observed when the entire LON was ablated (Fig. 4).

The overall integrity of the 5-HT system and the visual system morphology suggest that reduction of Rac function caused by these heteroallelic combinations does not have a pleiotropic effect on the nervous system development that would explain the disruption of the 5-HT arborization. Thus, although the present results do not provide a direct link between the LON-derived signal and Rac activation, they strongly suggest that a Rac-dependent signaling pathway is involved in the transduction of the signal provided by the LON for the development of this arborization.

Discussion

The dendritic arbor of the LNv and the termini of Rh5 and Rh6 photoreceptors overlap equally with the serotonergic arborization in the larval optic neuropil (Figs. 1 and 2). Ablation of two of these groups of neurons, the 3–4 Rh5 photoreceptors and the 4 LNv, did not disrupt the development of the 5-HT arborization (Figs. 3 and 5). However, ablation of Rh6-expressing fibers that originate from circa 8 retinular neurons had a drastic effect on the development of this 5-HT arborization (Fig. 4C). These observations are consistent with those found in *glass* (gl) mutants (Mukhopadhyay and Campos, 1995) and in *GMR-hid* larval brains (Fig. 4B) in which the LON is absent, suggesting that the Rh6 photoreceptors are uniquely required for the development of this serotonergic arborization.

The role of afferent activity in the development of postsynaptic partners is well documented in various systems (Miller and Kaplan, 2003; Sin et al., 2002; Van Aelst and Cline, 2004; Wong and Ghosh, 2002). Consistent with these observations is the finding that disruption of synaptic activity of the Rh6 fibers by targeted expression of tetanus-toxin light chain ("weak", TNT-E or "strong", TNT-G) caused reduction in the branching of the 5-HT arborization in the majority of specimens (Fig. 7D). Targeted expression of TNT completely eliminates evoked synaptic transmission and decreases spontaneous synaptic vesicle release by about 50% (Deitcher et al., 1998; Sweeney et al., 1995). Attenuation of electrical activity due to the expression, in Rh6 cells or in all photoreceptors, of the modified K^+ channel EKO did not have any effect on the development of the 5-HT arborization. It is possible that only partial suppression of excitability was achieved in these larvae, similar to what was observed for adult photoreceptors (White et al., 2001). However, behavioral assays carried out with third instar *GMR-GAL4/UAS-EKO* larvae revealed a significant reduction of the response to light compared to wild type larvae, suggesting that evoked potentials had been significantly suppressed. These observations are consistent with the finding that maintenance of larvae in the dark throughout development does not alter the morphology of the 5-HT projection in the larval optic neuropil (M. Mukhopadhyay and A.R. Campos, personal communication). Similarly, larvae carrying mutations in the *norpA* gene encoding the light-activated phospholipase C required for phototransduction did not reveal any disruption in the development of the 5-HT arborization (data not shown).

Taken together, these results suggest that proper branching of the 5-HT arborization in the larval optic neuropil does not depend on evoked synaptic activity of the Rh6-expressing fibers but may rely on spontaneous neurotransmitter release. Alternatively, expression of TNT disrupts this process independently of its effects on synaptic physiology. A requirement for synaptobrevin function for the proper expression of the neural cell adhesion molecule FasII has been reported in *Drosophila* (Baines et al., 2002; Hiesinger et al., 1999). Consistent with these observations is the demonstration that manipulation in the level of FasII expression mimics some aspects of the phenotypic consequences of synaptic suppression due to expression of TNT (Baines et al., 2002).

Thus, the partial disruption of the 5-HT arborization by targeted expression of TNT on the LON afferents, by comparison to the complete disruption observed when Rh6 photoreceptors are ablated, may be explained by either partial suppression of spontaneous neurotransmitter release or by a synaptic vesicle release-independent effect such as modification of FasII expression. Alternatively, Rh6 fibers may provide an activity-independent trophic support that potentiates and/or maintains activity-dependent processes, similar to what is found in other systems. For example, in the developing cortex Sema3A, neurotrophins and Slit interact to specify the basic morphology of cortical neurons. As development proceeds, the control of further growth and branching is shifted to activity-dependent mechanisms that rely on global and local increases of intracellular calcium (reviewed by Wong and Ghosh, 2002).

Our observations suggest a role for *Rac1* and/or *Rac2* signaling in the transduction of the signal provided by the LON (Fig. 8). These results are consistent with previous studies suggesting a requirement for the Rho family of small GTPases, notably, RhoA, Rac and Cdc42 in neuronal morphogenesis. For instance, Hakeda-Suzuki et al. (2002) and Ng et al. (2002) have

shown that *Drosophila* MB neurons mutant for *Rac1* and *Rac2* present defects in axon growth, guidance and branching. Interestingly, Rac has also been shown to be important for dendritic branching stability and morphogenesis of dendritic spines (reviewed in Govek et al., 2005; Luo, 2002; Van Aelst and Cline, 2004). For example, Lee et al. (2003) have shown that *Rac1* mutant *Drosophila* DA neurons developed fewer dendritic branches than wild type neurons in the third instar larval stage. Our observations are unique in *Drosophila* in that they suggest a role for Rac signaling in activity-dependent neuronal morphogenesis. Alternatively, Rac signaling may be required for the synaptobrevin-dependent developmental process discussed above and reported previously (Baines et al., 2002; Hiesinger et al., 1999).

Drosophila adults that have been reared in complete darkness since embryogenesis still display rhythmic behavior albeit not in synchrony with other individuals in the population (e.g. Sehgal et al., 1992). These observations support the notion that a circadian clock is assembled during embryogenesis and is functional as the larva hatches. Synchronization of the circadian clock or its entrainment can be achieved by light treatment as early as the end of the first instar, indicating that, in addition, photic input pathways are in place by the end of embryogenesis which are capable of resetting the pacemaker neurons (Malpel et al., 2004; Sehgal et al., 1992). Recent reports demonstrate a role for the larval visual system as a photic input pathway in entrainment during larval development (Malpel et al., 2004). Whether all or a subset of the 12 larval photoreceptors found in each of the two larval eyes are equally involved in this process has not yet been addressed. Similarly, it is not known whether the larval visual system functions as an entrainment input pathway equally throughout larval development. Relevant to these questions are our previous observations that indicate that Rh6-expressing photoreceptors are not involved in the basal response to light measured in our behavioral assay (Hassan et al., 2005). The results reported here demonstrate that these same photoreceptors (Rh6) are uniquely required for the induction of a 5-HT arborization that innervates the larval optic neuropil and that, it in turn, overlaps with the dendritic arborization of the main larval pacemaker neurons, the LNv. Thus, it is possible that the larval photoreceptor function, as a circadian input pathway, is modulated by their interaction with the 5-HT arborization that takes place during the third instar larval stage.

Several reports demonstrate the presence of circadian rhythms in the visual system of insects. Of note are the extensive analysis of Meinertzhagen and colleagues that established the existence in *Musca domestica* and *D. melanogaster* adults of circadian oscillation in the number of synapses between the outer photoreceptors (R1–R6) and the first order lamina interneurons L1 and L2 and in the diameter of L1 and L2 axons (Pyza and Meinertzhagen, 1993, 1995, 1999). These neuroanatomical changes are believed to be regulated by the neuromodulators 5-HT and pigment dispersing factor (PDF) present in two sets of neurons that innervate the optic lobe neuropiles of adult flies (Chen et al., 1999; Meinertzhagen and Pyza, 1996; Pyza and Meinertzhagen, 1996). This conclusion is supported by the observation that, in the *Caliphora* compound eyes, the circadian rhythmicity of the light-evoked response measured in electroretinograms (ERG) is affected by injection of 5-HT and PDF (Chen et al., 1999). A recent report detailing circadian oscillation in the larval response to light (Mazzoni et al., 2005) supports the hypothesis that the 5-HT arborization described here may be modulating larval visual system function.

Alternatively, the fact that 5-HT processes innervating the larval optic neuropil are found overlapping with the dendritic arborization of the LNv may suggest a direct modulation of the *Drosophila* larval pacemaker neurons. It has been shown that serotonin, in addition to its function in behavior, also plays a role in modulating circadian locomotor activity and heart rate in insects. For instance, it has reported that injection of the specific neurotoxin 5,7-DHT, which causes selective degeneration of serotonergic neurons, modified the level of locomotor activity and period of circadian rhythmicity in the blowfly (Cymborowski, 2003). Furthermore, it has been demonstrated that serotonin increases heart rate in *Drosophila* (Johnson et al., 1997, 2002), supporting the idea of a direct modulatory effect of this neurotransmitter on pacemaker cells.

Previous developmental analysis of the 5-HT arborization has shown that the contact between the LON and the serotonin process in the larval optic center occurs during late second–early third instar larval stage (Mukhopadhyay and Campos, 1995). After that, the 5-HT processes undergo further branching. These observations suggest that this serotonergic arborization may have a role in the function of the larval visual system during the third instar larval stage. Interestingly, it is towards the end of this stage that the larva becomes progressively less photophobic, attaining photo neutrality just before pupariation (Sawin-McCormack et al., 1995). Moreover, it has been shown that 5-HT is able to modulate the voltage dependency of K^+ channels in *Drosophila* adult photoreceptors (Hevers and Hardie, 1995; Kauranen and Weckstrom, 2004). Hence, it is possible that the innervation of the larval optic center by this 5-HT arborization plays a role in the modulation of the photobehavior that occurs during the foraging–wandering transition (Sawin-McCormack et al., 1995).

Acknowledgments

This work was supported by a Canadian Institute of Health Research grant (to A.R.C), grant number: MOP-12700. We thank Drs. Jay Hirsch and Maximiliano L. Suster for donation of stocks and Drs. Colin Nurse and Roger Jacobs for insightful comments and suggestions, which have contributed to the improvement of this article.

References

Baines, R.A., Seugnet, L., Thompson, A., Salvaterra, P.M., Bate, M., 2002. Regulation of synaptic connectivity: levels of Fasciclin II influence synaptic growth in the *Drosophila* CNS. J. Neurosci. 22, 6587–6595.

Busto, M., Iyengar, B., Campos, A.R., 1999. Genetic dissection of behavior: modulation of locomotion by light in the *Drosophila melanogaster* larva requires genetically distinct visual system functions. J. Neurosci. 19, 3337–3344.

Campos, A.R., Lee, K.J., Steller, H., 1995. Establishment of neuronal connectivity during development of the *Drosophila* larval visual system. J. Neurobiol. 28, 313–329.

Chen, B., Meinertzhagen, I.A., Shaw, S.R., 1999. Circadian rhythms in light-evoked responses of the fly's compound eye, and the effects of neuromodulators 5-HT and the peptide PDF. J. Comp. Physiol., A Sens. Neural Behav. Physiol. 185, 393–404.

Cymborowski, B., 2003. Effects of 5,7-dihydroxytriptamine (5,7-DHT) on circadian locomotor activity of the blow fly, *Calliphora vicina*. J. Insect Sci. 3, 14.

Deitcher, D.L., Ueda, A., Stewart, B.A., Burgess, R.W., Kidokoro, Y., Schwarz, T.L., 1998. Distinct requirements for evoked and spontaneous release of neurotransmitter are revealed by mutations in the *Drosophila* gene neuronal-synaptobrevin. J. Neurosci. 18, 2028–2039.

Fan, X., Labrador, J.P., Hing, H., Bashaw, G.J., 2003. Slit stimulation recruits Dock and Pak to the roundabout receptor and increases Rac activity to regulate axon repulsion at the CNS midline. Neuron 40, 113–127.

Furrer, M.P., Kim, S., Wolf, B., Chiba, A., 2003. Robo and Frazzled/DCC mediate dendritic guidance at the CNS midline. Nat. Neurosci. 6, 223–230.

Govek, E.E., Newey, S.E., Van Aelst, L., 2005. The role of the Rho GTPases in neuronal development. Genes Dev. 19, 1–49.

Green, P., Hartenstein, A.Y., Hartenstein, V., 1993. The embryonic development of the *Drosophila* visual system. Cell Tissue Res. 273, 583–598.

Grether, M.E., Abrams, J.M., Agapite, J., White, K., Steller, H., 1995. The head involution defective gene of *Drosophila melanogaster* functions in programmed cell death. Genes Dev. 9, 1694–1708.

Grueber, W.B., Jan, Y.N., 2004. Dendritic development: lessons from *Drosophila* and related branches. Curr. Opin. Neurobiol. 14, 74–82.

Hakeda-Suzuki, S., Ng, J., Tzu, J., Dietzl, G., Sun, Y., Harms, M., Nardine, T., Luo, L., Dickson, B.J., 2002. Rac function and regulation during *Drosophila* development. Nature 416, 438–442.

Hassan, J., Iyengar, B., Scantlebury, N., Rodriguez Moncalvo, V., Campos, A.R., 2005. Photic input pathways that mediate the *Drosophila* larval response to light and circadian rhythmicity are developmentally related but functionally distinct. J. Comp. Neurol. 481, 266–275.

Hevers, W., Hardie, R.C., 1995. Serotonin modulates the voltage dependence of delayed rectifier and Shaker potassium channels in *Drosophila* photoreceptors. Neuron 14, 845–856.

Hiesinger, P.R., Reiter, C., Schau, H., Fischbach, K.F., 1999. Neuropil pattern formation and regulation of cell adhesion molecules in *Drosophila* optic lobe development depend on synaptobrevin. J. Neurosci. 19, 7548–7556.

Hu, H., Marton, T.F., Goodman, C.S., 2001. Plexin B mediates axon guidance in *Drosophila* by simultaneously inhibiting active Rac and enhancing RhoA signaling. Neuron 32, 39–51.

Huang, E.J., Reichardt, L.F., 2001. Neurotrophins: roles in neuronal development and function. Annu. Rev. Neurosci. 24, 677–736.

Jan, Y.N., Jan, L.Y., 2003. The control of dendrite development. Neuron 40, 229–242.

Johnson, E., Ringo, J., Dowse, H., 1997. Modulation of *Drosophila* heartbeat by neurotransmitters. J. Comp. Physiol. [B] 167, 89–97.

Johnson, E., Sherry, T., Ringo, J., Dowse, H., 2002. Modulation of the cardiac pacemaker of *Drosophila*: cellular mechanisms. J. Comp. Physiol., B 172, 227–236.

Kauranen, M., Weckstrom, M., 2004. K+ channels and their modulation by 5-HT in *Drosophila* photoreceptors: a modelling study. Ann. Biomed. Eng. 32, 1580–1595.

Lee, A., Li, W., Xu, K., Bogert, B.A., Su, K., Gao, F.B., 2003. Control of dendritic development by the *Drosophila* fragile X-related gene involves the small GTPase Rac1. Development 130, 5543–5552.

Li, H., Chaney, S., Roberts, I.J., Forte, M., Hirsh, J., 2000. Ectopic G-protein expression in dopamine and serotonin neurons blocks cocaine sensitization in *Drosophila melanogaster*. Curr. Biol. 10, 211–214.

Li, Z., Aizenman, C.D., Cline, H.T., 2002. Regulation of rho GTPases by crosstalk and neuronal activity in vivo. Neuron 33, 741–750.

Luo, L., 2002. Actin cytoskeleton regulation in neuronal morphogenesis and structural plasticity. Annu. Rev. Cell Dev. Biol. 18, 601–635.

Malpel, S., Klarsfeld, A., Rouyer, F., 2002. Larval optic nerve and adult extra-retinal photoreceptors sequentially associate with clock neurons during *Drosophila* brain development. Development 129, 1443–1453.

Malpel, S., Klarsfeld, A., Rouyer, F., 2004. Circadian synchronization and rhythmicity in larval photoperception-defective mutants of *Drosophila*. J. Biol. Rhythms 19, 10–21.

Mazzoni, E.O., Desplan, C., Blau, J., 2005. Circadian pacemaker neurons transmit and modulate visual information to control a rapid behavioral response. Neuron 45, 293–300.

McAllister, A.K., 2000. Cellular and molecular mechanisms of dendrite growth. Cereb. Cortex 10, 963–973.

McAllister, A.K., 2001. Neurotrophins and neuronal differentiation in the central nervous system. Cell. Mol. Life Sci. 58, 1054–1060.

McAllister, A.K., Lo, D.C., Katz, L.C., 1995. Neurotrophins regulate dendritic growth in developing visual cortex. Neuron 15, 791–803.

Meinertzhagen, I.A., Pyza, E., 1996. Daily rhythms in cells of the fly's optic lobe: taking time out from the circadian clock. Trends Neurosci. 19, 285–291.

Miller, F.D., Kaplan, D.R., 2003. Signaling mechanisms underlying dendrite formation. Curr. Opin. Neurobiol. 13, 391–398.

Mukhopadhyay, M., Campos, A.R., 1995. The larval optic nerve is required for the development of an identified serotonergic arborization in *Drosophila melanogaster*. Dev. Biol. 169, 629–643.

Nassif, C., Noveen, A., Hartenstein, V., 2003. Early development of the *Drosophila* brain: III. The pattern of neuropile founder tracts during the larval period. J. Comp. Neurol. 455, 417–434.

Ng, J., Nardine, T., Harms, M., Tzu, J., Goldstein, A., Sun, Y., Dietzl, G., Dickson, B.J., Luo, L., 2002. Rac GTPases control axon growth, guidance and branching. Nature 416, 442–447.

Polleux, F., Morrow, T., Ghosh, A., 2000. Semaphorin 3A is a chemoattractant for cortical apical dendrites. Nature 404, 567–573.

Pyza, E., Meinertzhagen, I.A., 1993. Daily and circadian rhythms of synaptic frequency in the first visual neuropile of the housefly's (*Musca domestica L.*) optic lobe. Proc. Biol. Sci. 254, 97–105.

Pyza, E., Meinertzhagen, I.A., 1995. Monopolar cell axons in the first optic neuropil of the housefly, *Musca domestica* L., undergo daily fluctuations in diameter that have a circadian basis. J. Neurosci. 15, 407–418.

Pyza, E., Meinertzhagen, I.A., 1996. Neurotransmitters regulate rhythmic size changes amongst cells in the fly's optic lobe. J. Comp. Physiol., A Sens. Neural Behav. Physiol. 178, 33–45.

Pyza, E., Meinertzhagen, I.A., 1999. Daily rhythmic changes of cell size and shape in the first optic neuropil in *Drosophila melanogaster*. J. Neurobiol. 40, 77–88.

Rajan, I., Cline, H.T., 1998. Glutamate receptor activity is required for normal development of tectal cell dendrites in vivo. J. Neurosci. 18, 7836–7846.

Rajan, I., Witte, S., Cline, H.T., 1999. NMDA receptor activity stabilizes presynaptic retinotectal axons and postsynaptic optic tectal cell dendrites in vivo. J. Neurobiol. 38, 357–368.

Sawin-McCormack, E.P., Sokolowski, M.B., Campos, A.R., 1995. Characterization and genetic analysis of *Drosophila melanogaster* photobehavior during larval development. J. Neurogenet. 10, 119–135.

Sehgal, A., Price, J., Young, M.W., 1992. Ontogeny of a biological clock in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. U. S. A. 89, 1423–1427.

Sin, W.C., Haas, K., Ruthazer, E.S., Cline, H.T., 2002. Dendrite growth increased by visual activity requires NMDA receptor and Rho GTPases. Nature 419, 475–480.

Steller, H., Fischbach, K.F., Rubin, G.M., 1987. Disconnected: a locus required for neuronal pathway formation in the visual system of *Drosophila*. Cell 50, 1139–1153.

Sweeney, S.T., Broadie, K., Keane, J., Niemann, H., O'Kane, C.J., 1995. Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects. Neuron 14, 341–351.

Van Aelst, L., Cline, H.T., 2004. Rho GTPases and activity-dependent dendrite development. Curr. Opin. Neurobiol. 14, 297–304.

Van Vactor Jr., D., Krantz, D.E., Reinke, R., Zipursky, S.L., 1988. Analysis of mutants in chaoptin, a photoreceptor cell-specific glycoprotein in *Drosophila*, reveals its role in cellular morphogenesis. Cell 52, 281–290.

White, K., Tahaoglu, E., Steller, H., 1996. Cell killing by the *Drosophila* gene reaper. Science 271, 805–807.

White, B.H., Osterwalder, T.P., Yoon, K.S., Joiner, W.J., Whim, M.D., Kaczmarek, L.K., Keshishian, H., 2001. Targeted attenuation of electrical activity in *Drosophila* using a genetically modified K(+) channel. Neuron 31, 699–711.

Whitford, K.L., Marillat, V., Stein, E., Goodman, C.S., Tessier-Lavigne, M., Chedotal, A., Ghosh, A., 2002. Regulation of cortical dendrite development by Slit–Robo interactions. Neuron 33, 47–61.

Wong, R.O., Ghosh, A., 2002. Activity-dependent regulation of dendritic growth and patterning. Nat. Rev., Neurosci. 3, 803–812.

Yamashita, T., Tucker, K.L., Barde, Y.A., 1999. Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. Neuron 24, 585–593.

Zipursky, S.L., Venkatesh, T.R., Teplow, D.B., Benzer, S., 1984. Neuronal development in the *Drosophila* retina: monoclonal antibodies as molecular probes. Cell 36, 15–26.